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Transfer of Nonselectable Genes into Mouse Teratocarcinoma Cells and Transcription of the Transferred Human β-Globin Gene

ERWIN F. WAGNER AND BEATRICE MINTZ*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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Teratocarcinoma (TCC) stem cells can function as vehicles for the introduction of specific recombinant genes into mice. Because most genes do not code for a selectable marker, we investigated the transformation efficiency of vectors with a linked selectable gene. In one series, TCC cells first selected for thymidine kinase deficiency were treated with DNA from the plasmid vector PtkHβ1 containing the human genomic β-globin gene and the thymidine kinase gene of herpes simplex virus. A high transformation frequency was obtained after selection in hypoxanthine-aminopterin-thymidine medium. Hybridization tests revealed that the majority of transformants had intact copies of the human gene among three to six total copies per cell. These were associated with cellular DNA sequences as judged from the presence of additional new restriction fragments and from stability of the sequences in tumors produced by injecting the cells subcutaneously. Total polyadenylate-containing RNA from cell cultures of two out of four transformants examined showed hybridization to the human gene probe: one RNA species resembled mature human β-globin mRNA transcripts; the others were of larger size. In differentiating tumors, various tissues, including hematopoietic cells of TCC provenance could be found. In a second model set of experiments, wild-type TCC cells were used to test a dominant-selection scheme with pSV-*gpt* vectors. Numerous transformants were isolated, and their transfected DNA was apparently stably integrated. Thus, any gene of choice can be transferred into TCC stem cells even without mutagenesis of the cells, and selected cell clones can be characterized. Cells of interest may then be introduced into early embryos to produce new mouse strains with predetermined genetic changes.

Mouse teratocarcinoma (TCC) stem cells from two transplant lines (5, 18) and a unique culture line (17) can undergo completely normal development, including formation of functional germ cells (26a), after they are injected into early embryos. Therefore, the cells of this in vitro line could serve as vehicles for the introduction of predetermined genetic changes into mice and establishment of new genetic strains (15, 16). These changes may begin with selection in culture of TCC cells with a specific nuclear mutation (8), mitochondrial mutation (31), or an exogenously supplied gene that has been produced by recombinant DNA technology (22) and may be in native or modified form. Such modifications, or fortuitous integration in different chromosomal sites, could, after differentiation of the cells in embryos, provide useful clues to developmental regulation of gene expression in an entire intact organism.

Many recombinant genes of interest would not, however, be selectable, either because a suitable selection (or screening) method does not exist or because that gene is not expressed in cultured cells. Globin genes are an example. Three general schemes have been devised to circumvent this difficulty: In two of them (cotransfer with a simultaneously added but unlinked selectable gene [34] or linkage with a selectable gene in the same vector [14]) a mutant recipient cell is required. Thus, a mutagenized subline deficient in thymidine kinase activity would provide the possibility that transformants that had taken up the thymidine kinase gene of, say, herpes simplex virus (HSV) (and perhaps the accompanying unselectable gene) could be isolated by selection in hypoxanthine-aminopterin-thymidine (HAT) medium. The third general scheme, involving novel dominant-selection vectors (19, 20), allows wild-type recipient cells to be used, thereby obviating the initial mutagenesis and prolonged selection steps during

which undesirable ancillary genetic changes might occur.

In an initial study (22), we demonstrated the feasibility of introducing the unselectable human β-globin gene into the TCC lineage by cotransfer with the HSV thymidine kinase gene into thymidine kinase-deficient (tk^-) cells. Although the transformations were stable, they occurred at a frequency appreciably lower than that for mouse L tk^- cells, and the tk^- cells were highly aneuploid. In the present study, we investigated, using a TCC tk^- line of quasi-normal karyotype, the efficiency of linked transfer of the same two genes; we also examined the general applicability to TCC cells of dominant-selection, by testing one of the recently designed dominant-acting vectors. We report that both the linked transfer and dominant-selection methods yield stable TCC transformants with high efficiency; and that the cells retain the capacity to differentiate into a large variety of specialized types, including hematopoietic cells, in tumors formed by cell inoculation into mice. Moreover, cultured cells from the linked transfer experiment show evidence of human globin-specific transcripts.

MATERIALS AND METHODS

Cell lines. Of the two wild-type TCC cell lines in the present study, one of them, designated F22CAC and used in the linked transfer experiment, had a common origin with the karyotypically normal and developmentally totipotent METT-1 cell line (17), whereas the other, which was used in the dominant-selection experiment, was the METT-1 line itself.

The F22CAC line was derived from the same primary tumor (F22, originating from a grafted embryo of the 129/SV inbred strain) as the METT-1 line and shared the same early explant history (17). However, after a few passages in vitro, some of the cultured cells were injected intraperitoneally into syngeneic hosts to obtain a parallel in vivo ascites line, whereas most of the cells continued to be passaged in vitro and eventually led to the stable METT-1 line. The ascites conversion probably involved strong selection among an initially small number of surviving cells. After four ascites passages, some cells were reexplanted and cultured for seven more passages in vitro before being subjected to mutagenesis. (This new in vitro source was used because no METT-1 cells of known karyotype and developmental capacity were in culture at that time, and the frozen batches were still incompletely characterized.) No karyotype information was obtained on the wild-type F22CAC cells. However, when the ascites cells of origin were examined karyologically after seven continuous in vivo passages, some abnormalities were found (unpublished data). It is therefore likely that even before mutagenesis (see below) the F22CAC cells were not entirely normal karyotypically.

All TCC cells were grown in the medium described (17) except for changes specified for selection. No feeder cell layer was present.

Mutagenesis and selection. TCC cells of the F22CAC line were seeded at a density of 5×10^6 cells per 75-cm^2 tissue culture flask, and 3 h later, the mutagen ICR 191 (a gift from Richard M. Peck of this institute) was added at 0.5 μg/ml. After 20 h, the cells were trypsinized, divided equally into four tissue culture flasks (150 cm^2), and allowed to grow without selection for 7 days The cells were then transferred to medium containing 3 μg of bromodeoxyuridine (BrdUrd) per ml. Resistant clones were isolated after 3 weeks and maintained in the presence of 30 μg of BrdUrd per ml for about 2 months before they were used for experiments.

Tumor formation. Subcutaneous tumors were produced in syngeneic female recipients by inoculating 10^7 cells suspended in 0.25 ml of phosphate-buffered saline. Solid tumors developed within 3 weeks. Parts of all tumors were fixed for histological examination; some were retransplanted by subcutaneous injection of 6 to 10 small pieces, and some tumor material was used for DNA and RNA preparations and for enzyme assays.

Transformation experiments. Transformation in most instances was performed as described before (22, 32). The recombinant plasmid, PtkHβ1, was kindly provided by Tom Maniatis and prepared by Pamela Mellon at the California Institute of Technology. It contains a 7.6-kilobase (kb) *Hin*dIII fragment of the human β-globin gene, isolated from a genomic λ clone, HβG3 (10), and cloned into the *Hin*dIII site of Ptk (4). To each plate of attached tk^- cells derived from the F22CAC line, a calcium phosphate-DNA precipitate prepared with 6 μg of undigested PtkHβ1 DNA was added, either in the presence of 20 μg of high-molecular-weight DNA obtained from salmon sperm or without carrier DNA. After 9 h, the cells were treated for 30 min with medium containing 10% dimethyl sulfoxide (12), washed once, and incubated for an additional 24 h. The medium was then replaced with HAT medium (0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine). Colonies of transformants were isolated after 8 to 14 days.

Transformation experiments involving wild-type cells of the developmentally totipotent METT-1 line were performed with 15 μg of undigested pSV2-*gpt* DNA per plate as recently described (19, 20). The recombinant plasmid, pSV2-*gpt*, was kindly provided by Paul Berg of Stanford University. It contains a 1.0-kb fragment of the bacterial xanthine-guanine phosphoribosyltransferase gene (*Ecogpt*), several regulatory elements of simian virus 40, and a 2.3-kb fragment of the plasmid pBR322 (19). After the cells were incubated for 6 h with the calcium phosphate-DNA precipitate, the medium was removed, and 15% glycerol in isotonic *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline was added, at 5 ml per plate, for 3 min at 37°C as described before (21). The cells were washed once, incubated for 2 days at 37°C without selection, trypsinized, and seeded on 100-mm plates in medium containing 10% fetal calf serum, 1.4 mM xanthine, 0.11 mM hypoxanthine, 41 μM thymidine, 4.5 μM aminopterin, and 77 μM mycophenolic acid. Colonies of transformants were isolated after 6 to 10 days and maintained under selective conditions before further use.

Isolation of transformed-cell DNA and blot hybridizations. High-molecular-weight DNA was isolated from cultured cells and tumor tissue by standard proce-

dures. Genomic DNA (15 μg) was digested with restriction endonucleases, and the product was electrophoresed on horizontal 1% agarose slab gels. The fragments were transferred to nitrocellulose sheets, hybridized, and washed as described before (27, 28, 30). DNAs used as probes were labeled with ^{32}P by nick translation (24); gel-purified fragments were obtained by electroelution.

Isolation of RNA and RNA filter hybridizations. Total RNA was isolated from cultured TCC cells and from tumor tissue after homogenization and successive extractions with phenol at pH 5.1, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1) as described before (34). Total polyadenylate [poly(A)]-containing RNA was isolated by oligodeoxythymidylate-cellulose chromatography (2) and was electrophoresed through 1% agarose gel containing 6% formaldehyde, 20 mM morpholinepropanesulfonic acid (MOPS) buffer, 5 mM sodium acetate, and 1 mM EDTA. The concentration of total poly(A)-containing RNA in each slot was 10 μg; electrophoresis was performed at 30 V for 12 h at room temperature. The separated RNAs were transferred from the gel to nitrocellulose paper (0.1-μm pore size) (28), hybridized, washed, and autoradiographed as described above for the DNA blots.

Assay of thymidine kinase activity. The preparation and assay of cell and tissue extracts for thymidine kinase was performed as described before (22, 23).

Metaphase cell preparations and karyotype analyses were done essentially as described before (5).

RESULTS

Isolation of a tk$^-$ TCC cell line. From wild-type F22CAC cells exposed to the mutagen ICR 191, three colonies (TCC-tk$^-$-1, -2, and -3) resistant to BrdUrd were recovered and were maintained in the presence of BrdUrd for about three months before they were further characterized. Two of the mutant cell clones, TCC-tk$^-$-2 and -3, proved to be unstable and reverted to tk$^+$ at a high frequency; the remaining BrdUrd-resistant clone, TCC-tk$^-$-1, had a very stable tk$^-$ phenotype (Table 1). When thymidine kinase activity was measured in the mutant clones, the level of residual enzyme activity was low (Table 1). The ability of the tk$^-$ cells to differentiate was determined by producing tumors in syngeneic mice. Histological examination showed that, in addition to undifferentiated stem cells, the tumor derived from TCC-tk$^-$-1 cells contained many differentiated tissues, including neural cells, muscle, cartilage, fat, and various types of epithelia. The thymidine kinase activity from the tumor tissue was also low (Table 1). Thus, mutagenesis and selection had not diminished the potential of the stable mutant TCC stem cells to differentiate under these conditions.

Karyotype analysis of the ICR 191-treated (unselected) cells disclosed that the cells were still chromosomally XX (female) as was apparently the case in the tumor of origin (17) and that there was only one departure from normalcy: a trisomy of chromosome 11. (As already stated, information was not available on the karyotype of the F22CAC wild-type line.) The TCC-tk$^-$-1 selected cell clone had a modal chromosome number of 40 (84%) and a range of 39 to 41 chromosomes. It lost one X chromosome and acquired one rearranged chromosome, which in some metaphase spreads was identifiable as part of chromosomes 3 and 8.

TABLE 1. Characterization of thymidine kinase-deficient TCC clones in relation to wild-type TCC cells

Cell source	Thymidine kinase activity[a] (% of wild type)	Reversion frequency[b]
Wild type[c]	143.0 (100)	
Selected[d]		
TCC-tk$^-$-1	2.4 (1.7)	$<5 \times 10^{-9}$
Tumor from TCC-tk$^-$-1	4.9 (3.4)	
TCC-tk$^-$-2	5.4 (3.8)	5×10^{-5}
TCC-tk$^-$-3	3.0 (2.1)	3×10^{-7}

[a] Milliunits per milligram of protein in the assay described previously (22).

[b] Determined by seeding 2×10^8 cells in HAT medium after growth in BrdUrd (30 μg/ml) for about 3 months.

[c] Nonmutagenized TCC cultured stem cells of the F22CAC cell line (see the text).

[d] After treatment of wild-type TCC cells with the mutagen ICR 191 at 0.5 μg/ml for 20 h, three tk$^-$ clones (TCC-tk -1, -2, and -3) were selected for BrdUrd resistance.

Linked transfer of the human β-globin gene into tk$^-$ TCC cells. A large number of HAT-resistant colonies were obtained after TCC-tk$^-$-1 cells were treated with DNA from the plasmid vector PtkHβ1, which includes both the HSV tk gene and the adult genomic human β-globin gene. In the absence of any carrier DNA, about 5 to 10 colonies per 5×10^5 cells were found, whereas in the presence of 20 μg of salmon sperm DNA, the transformation frequency was three times higher. Thus, the efficiency of transformation with TCC-tk$^-$-1 stem cells in these experiments is very much higher than the efficiency achieved in our earlier experiments with extremely aneuploid tk$^-$ TCC cells (22).

Identification of human β-globin DNA in the transformed TCC cells and their differentiated tumor derivatives. Cellular DNAs from seven individual HAT-resistant colonies (out of approximately 250 obtained) were analyzed for the presence of an intact human β-globin gene. High-molecular-weight DNA was digested with the restriction endonuclease *Pst*I, blotted onto nitrocellulose paper, and hybridized with a ^{32}P-

labeled 7.6-kb *Hin*dIII fragment encoding the entire human genomic β-globin gene. The DNAs of all seven colonies yielded as the major band the diagnostic 4.4-kb *Pst*I fragment (Fig. 1). When the DNA from the untransformed tk⁻ cell line was analyzed, no hybridization to the mouse β-globin genes was observed. There was no major difference in the band pattern from cell clones derived without carrier DNA (Fig. 1, lanes 1 to 4) as compared with those obtained in the presence of salmon sperm DNA (Fig. 1, lanes 5 to 7). Cell clones 1, 4, and 7 also displayed the neighboring (downstream) 4.0-kb *Pst*I fragment, thereby suggesting the complete conservation of the 7.6-kb human β-globin gene fragment in at least some of the introduced copies. In clones 2, 5, and 6, this 4.0-kb fragment was missing (Fig. 1) and was most likely lost during the integration event. Additional *Pst*I bands larger than the 4.4-kb fragment were found in transformants 3 and 4; this can be explained by a loss in one *Pst*I region and utilization of *Pst*I sites in adjacent cellular DNA. The number of copies of human β-globin genes was small (in the range of three to six copies per transformed cell genome), as estimated from the control lane containing a known amount of *Pst*I-cleaved PtkHβ1 DNA (Fig. 1).

The critical question of stable retention of the human β-globin gene during TCC cell differentiation in vivo in the absence of selection was examined in solid tumors, produced in syngeneic hosts, from cells of transformants 1, 2, 3, and 5. The restriction fragment profiles of the DNAs from these tumors did not change (under the same test conditions as described for DNAs of the cell lines) even when the tumors were maintained in vivo for about 3 months in three consecutive transplant hosts (Fig. 1, lane 1b). Therefore, the human β-globin sequences were apparently stably incorporated into the mouse genome without rearrangement during tissue differentiation.

Identification of human β-globin-specific transcripts in transformed TCC cells. Total poly(A)-containing RNA was isolated from four cell clones containing the human β-globin gene (1, 2, 3 and 5) to test for transcriptional activity of that gene. By using the procedures described before (28), RNA that had been electrophoresed and transferred to nitrocellulose paper was hybridized with the ^{32}P-labeled 7.6-kb human β-globin DNA fragment. A discrete 10S RNA species which comigrated with human globin mRNA was detected in transformant 1 (Fig. 2). In transformant 5, which was obtained by transfor-

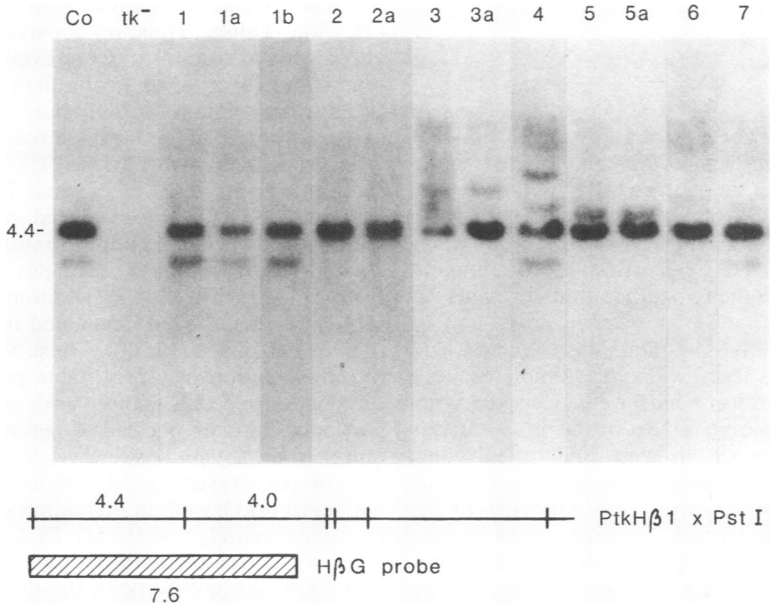

FIG. 1. Identification of human β-globin gene sequences in cultured stem cells of seven transformed TCC clones and four of their in vivo tumor derivatives. High-molecular-weight DNA was digested with *Pst*I and hybridized to the ^{32}P-labeled 7.6-kb *Hin*dIII fragment of PtkHβ1 (see text for details). Co, 200 pg of *Pst*I-digested PtkHβ1 DNA (~6.6 gene equivalents); tk⁻, the thymidine kinase-deficient mutant TCC-tk⁻-1; 1 to 4, independent transformants obtained without carrier DNA; 5 to 7, transformants from an experiment with carrier DNA. DNA from a tumor derived in the first host is specified by the suffix a; that in the third host is specified by the suffix b. All seven transformants contained the diagnostic 4.4-kb fragment spanning the human β-globin coding region; some also had the neighboring 4.0-kb fragment (1, 4, and 7).

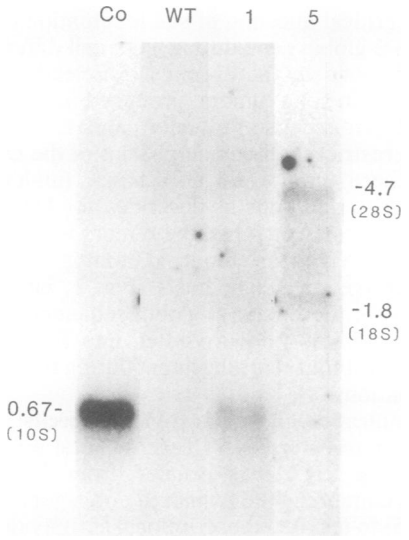

FIG. 2. Identification and size of human β-globin-specific transcripts in cultured stem cells of two transformed TCC clones. Total poly(A)-containing RNA was hybridized with the ^{32}P-labeled 7.6-kb *Hin*dIII fragment of PtkHβ1 DNA. The positions of 28S and 18S RNAs on the gel were determined optically after staining with ethidium bromide. Co, 1 ng of purified poly(A)-containing RNA from human cord blood cells; WT, 10 μg of total poly(A)-containing RNA from wild-type TCC cells; 1 and 5, 10 μg of total poly(A)-containing RNA from the two independent transformants.

mation with carrier DNA in addition to PtkHβ1 DNA, two RNA species were detected; both were larger than the mature mRNA (Fig. 2). No hybridization was observed in lanes with total poly(A)-containing RNA from either the wild-type (F22CAC) TCC cells, from the tk$^-$ mutant TCC line, or from two other transformants (2 and 3).

When total poly(A)-containing RNA was isolated from the corresponding tumor tissues, strong hybridization bands were observed with the human β-globin DNA probe in all transformants. These signals were undoubtedly due to cross-hybridization with endogenous mouse globin RNA, which would be expected to be present in vascularized, growing tumor tissue. Mouse globin transcripts would, however, be absent from TCC stem cells growing in culture, as was found when RNA from such cells was hybridized to a mouse β-globin probe (A. Pellicer, E. F. Wagner, R. Axel, and B. Mintz, unpublished data). Thus, the available evidence points to the presence of human β-globin-specific RNA sequences in these cultures. The use of more narrowly defined probes, in conjunction with the S1 nuclease assay (3), should elucidate the structure of these human globin-specific RNAs.

Tissue differentiation in tumors containing the human β-globin gene. Tumors containing intact sequences of the human β-globin gene (Fig. 1) were examined histologically to learn whether differentiation had been impaired by mutagenesis and by two steps of selection (in BrdUrd and in HAT). Samples from four independent clones (1, 2, 3, and 5) included not only the TCC stem cells, but also a wide variety of differentiated tissues, similar to those in tumors from the untransformed mutant parent line (TCC-tk$^-$-1). Among the most prominent tissues were muscle, cartilage, and neural formations, and some bone and fat cells. The additional presence of hematopoietic cells in some of the tumors was especially noteworthy inasmuch as those cells, or their more differentiated erythroid derivatives, would ultimately be the targets for any normally regulated expression of the foreign globin gene. Even under these limiting (tumorigenic) circumstances, primitive erythroblasts, granulocytes, and megakaryocytes (Fig. 3) were formed. They could be attributed to the TCC lineage inasmuch as cells of these immature stages are absent from the host's circulation.

Karyotypes of two of the transformed cell culture lines (1 and 2) showed no obvious alterations when compared with the parental (TCC-tk$^-$-1) line. Thus, no apparent changes in chromosomal composition or developmental potential had supervened, according to these criteria, during isolation of the mutant recipient line and selection-mediated transformation.

Transformation of wild-type TCC cells by dominant-selection. A set of plasmid DNA vectors carrying the selectable bacterial gene for xanthine-guanine phosphoribosyltransferase (*Ecogpt*) and different regulatory sequences from simian virus 40, in addition to pBR322 sequences, have been developed in the laboratory of Paul Berg (19, 20). These vectors allow the introduction of any suitable gene into cultured wild-type cells without prior selection for a particular mutant derivative, as would be required in the thymidine kinase selection scheme. To compare results of this mode of selection with selection based on thymidine kinase activity, approximately 5×10^5 METT-1 cells (17) per plate were treated with 15 μg of DNA from pSV2-*gpt*, the prototype of the dominant-selection vectors (20). After 2 days without selection, mycophenolic acid and aminopterin were added to the medium, which was also supplemented simultaneously with xanthine, hypoxanthine, and thymidine. Cells that received no DNA or only salmon sperm DNA did not survive under the selective conditions; cultures treated with DNA from pSV2-*gpt* yielded 15 to 25 surviving

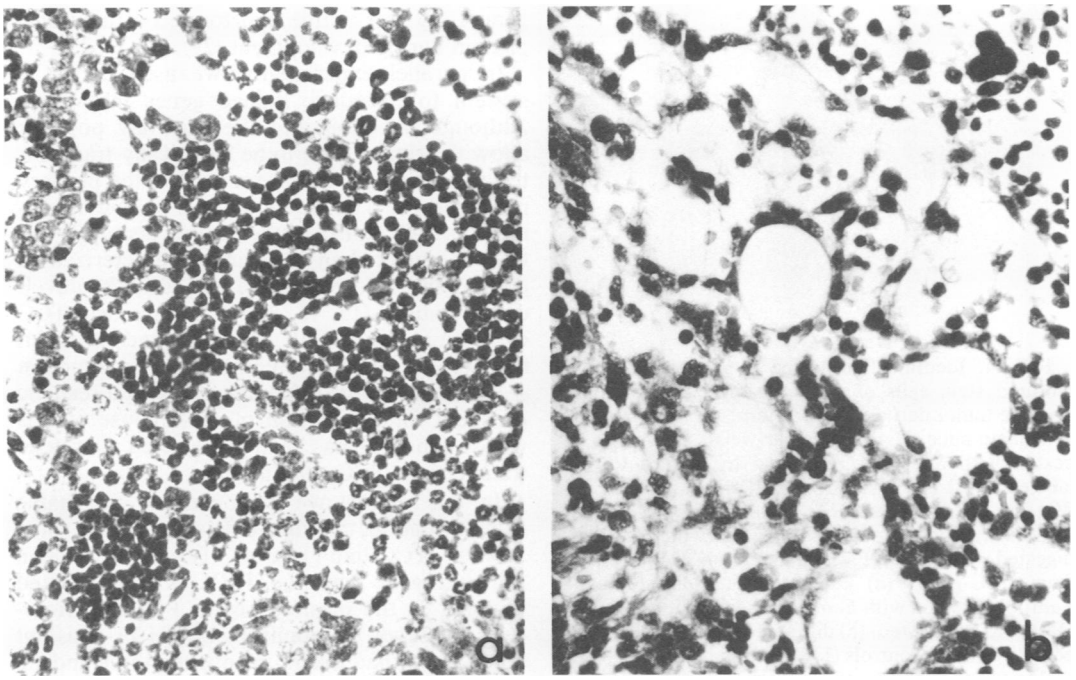

FIG. 3. Hematopoietic tissue differentiating in a tumor produced by in vivo inoculation with mouse TCC cells (of cell clone 2) containing the human β-globin gene. (a) Darkly staining patches of nucleated erythrocytes and lighter patches of granulocytes; (b) an area including conspicuous fat formation (as in bone marrow), a trio (upper right) of hematopoietic stem cells, and nucleated erythrocytes. ×400.

colonies per 5×10^5 cells plated. This transformation frequency was comparable to the results from the thymidine kinase selection experiment described here. We also obtained a similar transformation frequency in a separate experiment in which another *gpt* vector, pSV5-*gpt*, was used.

Physical state and number of vector-*gpt* DNA copies in transformed TCC cells. The number of vector-*gpt* DNA copies and their location and stability were examined by blot hybridization in two randomly picked pSV2-*gpt* transformants (TCC-*gpt*-1, -2), and one tumor derivative. Figure 4 shows the results of hybridizations of undigested and of *Sac*I- or *Eco*RI-cleaved cell DNAs from these transformants, when ^{32}P-labeled pSV2-*gpt* DNA was used as the probe. *Sac*I does not cleave pSV2-*gpt* DNA; therefore, each hybridizing band represents at least one copy of all or part of the vector DNA embedded in the cellular DNA, as judged from the intensity and size of the hybridizing fragments. *Eco*RI cuts the vector DNA once; consequently, a single, integrated, uninterrupted vector *gpt* copy should yield two bands, each containing flanking cellular DNA sequences. A tandemly repeated DNA arrangement with the intact *Eco*RI sites would yield at least one band containing full-length vector *gpt* DNA.

The results clearly show that the TCC stem cells have incorporated one to three vector *gpt* copies; all hybridizable material was associated with high-molecular-weight DNA when undigested DNA was separated in an agarose gel (Fig. 4). *Sac*I digests gave one hybridizable fragment in both transformants, and only one labeled band was obtained when the DNA of TCC-*gpt*-2 was cleaved with *Eco*RI. This can be explained if integration of the vector DNA occurred near its *Eco*RI restriction site or if that site became deleted during integration. No change in the position of the labeled band was visible in *Sac*I-digested DNA from a tumor derived from the TCC-*gpt*-1 cell clone as compared with the DNA from the cultured cells (Fig. 4, lanes b and e). However, an additional fragment was obtained in the *Eco*RI-digested DNA of this tumor that comigrated with the linear form of pSV2-*gpt* DNA and was missing from the DNA of the cultured cells. This band might have arisen by internal duplication of vector DNA sequences and a change in the relative position of *Sac*I sites.

The transformed phenotype is apparently stable, and it is likely that the *gpt* gene continued to be expressed under selective pressure. Histological examination of a tumor sample revealed that

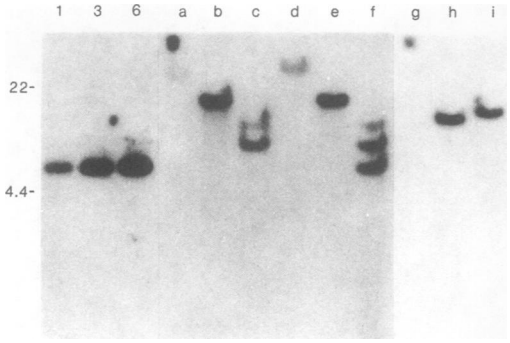

FIG. 4. Identification of pSV2-*gpt* sequences in cultured stem cells of two transformed TCC clones and one tumor derived by subcutaneous inoculation in syngeneic mice. High-molecular-weight DNA was digested with *Sac*I or *Eco*RI and hybridized to ^{32}P-labeled pSV2-*gpt* DNA. Size markers were from *Hin*dIII-cleaved phage λ DNA. Lanes a to c, TCC-*gpt*-1 DNA: (a) undigested, (b) digested with *Sac*I, (c) digested with *Eco*RI. Lanes d to f, tumor DNA derived from TCC-*gpt*-1: (d) undigested, (e) digested with *Sac*I, (f) digested with *Eco*RI. Lanes g to i, TCC-*gpt*-2 DNA: (g) undigested, (h) digested with *Sac*I, (i) digested with *Eco*RI. Controls (1, 3, and 6) are *Eco*RI digests of 15, 45, and 90 pg, respectively, of pSV-*gpt* DNA, equivalent to 1, 3, and 6 vector DNA copies per cell.

the stem cells retained the capacity to form an array of differentiated tissues similar to those in tumors from the wild-type METT-1 cells (17). Karyotype analysis of the transformed clone TCC-*gpt*-1 demonstrated that, in the majority of the cells, one chromosome was longer than in the karyotypically normal parental cells.

DISCUSSION

The introduction of strategically designed pure genes into laboratory mammals would increase our ability to analyze in vivo the developmental regulation of gene expression and to determine the molecular basis of many genetic diseases. We have recently reported (29) that cloned, recombinant genes that have been injected into a pronucleus of fertilized mouse eggs can be retained in an intact form throughout development and can also become functional. The DNA vector PtkHβ1 used in that study (and also in one of the experiments reported here) contained the adult human genomic β-globin gene (10), which would normally exhibit tissue-specific gene expression, and the HSV tk gene (4), which is capable of constitutive expression of the enzyme product in mammalian cells. Five (15%) of the animals were found to have multiple copies of the foreign genes, and, in one case, HSV-specific thymidine kinase enzyme activity was found, thereby indicating that at least one gene copy was active and formed a functional protein during development (29).

In parallel experiments, we used the TCC system to introduce cloned genes into mice. Although injection into the egg may possibly allow the donor gene to be mitotically transmitted to all somatic and germ cells in the primary recipient, the TCC route has the unique advantage that cell clones of the desired genotype can first be selected in vitro and characterized in great detail before they are placed in the developing embryo (15, 16, 22). Inasmuch as a TCC cell culture line, METT-1 (17), has recently been found to be capable of contributing to the germ cell lineage in vivo (26a), there is now a realistic prospect for production of new mouse strains whose genotype has been preselected after gene transfer into TCC cells in vitro.

In this report, we demonstrated the stable transformation, at high efficiency (10^{-5}), of a new tk$^-$ TCC line (TCC-tk$^-$-1) with the human β-globin gene and the HSV tk gene ligated together in the plasmid vector PtkHβ1. Moreover, of seven randomly picked HAT-resistant colonies, all had taken up between three and six copies of the nonselectable human β-globin gene. Thus, both the overall transformation frequency and the frequency of uptake of the unselectable globin gene were considerably higher than in our previous study in which the unselectable gene was cotransferred with the unlinked selectable gene (22). In the present cases, the *Pst*I-digested cellular DNAs of all seven transformants contained the diagnostic 4.4-kb fragment spanning the entire human β-globin coding region (Fig. 1). In addition, new *Pst*I fragments, not present in *Pst*I-digested PtkHβ1 DNA, were seen in clones 3 to 5, whereas in clones 2 and 3 and in 5 and 6, the downstream 4.0-kb segment which is present in the vector was missing (Fig. 1). When the separated pieces from the same *Pst*I digest were tested for hybridization with an HSV tk probe, each independent transformant showed at least one additional hybridizing band that was absent in the control *Pst*I digest of PtkHβ1 DNA (data not shown). The missing or additional *Pst*I bands suggest an integration event and they can be explained by a loss of one *Pst*I site in the vector DNA and cleavage in adjacent cellular sequences.

Evidence for human β-globin-specific RNA transcripts was found at a low level (5 to 10 molecules per cell) in two out of four TCC cell clones that contained the human gene. In one case (1), it appears that mature mRNA was formed, whereas the second clone (5) showed only two larger poly(A)-containing RNA species (Fig. 2). The initial product of transcription of the human β-globin gene in erythroid cells has

been described as a 1.8-kb poly(A)-containing RNA species (11). One globin RNA of transformant 5 is approximately 2.0 kb long and could, therefore, be a true precursor RNA. The second larger RNA might have been initiated at a downstream promoter in pBR322 or in adjacent DNA sequences, including those in the carrier DNA, which is probably integrated together with the vector DNA (23). A detailed analysis and verification of the 5′ and 3′ ends of the RNA with the method of Berk and Sharp (3) would be necessary to support these interpretations and to compare these initial observations with β-globin mRNA production in L cells after transformation with the rabbit β-globin gene (34, 14, 9). In another report (1) dealing with microinjection of human β-globin DNA into mouse fibroblasts, the results were taken as suggestive of the synthesis of some human mRNA sequences; however, no data were provided concerning the size or structure of that RNA.

The fact that some of our transformants did not show any detectable human mRNA and some did, albeit at a low level, may signify that the undifferentiated stem cells lack factors needed to promote expression of a gene normally expressed only in a specific cell type. Work with the F-9 line of TCC cells and simian virus 40 has led to the view that the stem cells lack such factors as RNA-processing enzymes (26) or will only form a functional RNA and protein when they are induced to differentiate (13). Whether some cells are actually undergoing some degree of differentiation in our cultures cannot be readily ascertained. The capacity for the foreign globin gene to be normally expressed will ultimately have to be evaluated in erythroid cells of mice. To some extent, relevant information may also be obtainable from hematopoietic tissue formed in tumors (Fig. 3) or in differentiating (organ-type) cultures of TCC cells as observed in cultures of the METT-1 line (17) and another line, PCC3/A/1 (6, 7).

Two requirements for experiments involving introduction of genes are that they remain stable in vivo in the absence of selective pressure and that prior selection and prolonged growth in culture do not alter their capacity to differentiate in vivo. Stability of the donor globin gene was shown in tumors from transformants 1, 2, 3, and 5. Even after they were retransplanted over a period totalling three months, without selection, the globin band pattern remained unchanged (Fig. 1, lane 1b). These tumors, in particular tumor 2, had various types of differentiated tissues, including hematopoietic cells.

A principal disadvantage of the thymidine kinase selection system is the necessity for first deriving a stable tk^- mutant cell line as a recipient for the transforming DNA. Dominant genetic markers, such as the *E. coli gpt* gene in the pSV-*gpt* vectors (19, 20), offer a solution inasmuch as it should be possible to apply them to unmutagenized wild-type cells. Stable transformation with DNA from the plasmid vector pSV2-*gpt* was in fact realized with wild-type TCC cells of the METT-1 line (Fig. 4). It should therefore be possible to utilize vectors of this type for transfer of cloned globin (or other unselectable) genes into TCC cells by ligating them in the same vector.

Normalcy and stability of the karyotype of TCC cells is likely to be a significant factor in retention of developmental totipotency after the cells are injected into blastocyst-stage embryos (5, 18). It is therefore important that deleterious changes not occur during selection in culture. Karyotype analysis was not a primary objective in the present investigations and only limited information was obtained. Nevertheless, the results appear promising. The karyotypes of two randomly chosen cell clones, isolated by HAT medium selection and containing the human β-globin (and HSV tk) gene, showed no apparent change as compared with the parent tk^- line (TCC-tk^--1). In the dominant-selection series, karyotypes were analyzed in one of many gpt^+ transformants (TCC-*gpt*-1) and showed a change in only one chromosome.

A question of considerable interest for further in vivo studies of TCC cell differentiation in embryos is whether an introduced foreign gene resides at a specific chromosomal location as has been found for a variant human growth hormone gene transferred into cells of a rat liver line (25). In our globin-gene transformants, preliminary data, based on statistical distribution of silver grains in in situ hybridization tests of metaphase spreads, suggest that a chromosomal integration event has occurred; however, a specific site has not yet been determined (E. F. Wagner, A. S. Henderson, S. Ripley, and B. Mintz, unpublished data).

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LITERATURE CITED

1. **Anderson, W. F., L. Killos, L. Sanders-Haigh, P. J. Kretschmer, and E. G. Diacumakos.** 1980. Replication and

expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **77**:5399–5403.
2. **Axel, R., P. Feigelson, and G. Schutz.** 1976. Analysis of the complexity and diversity of mRNA from chicken liver and oviduct. Cell **7**:247–254.
3. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
4. **Colbere-Garapin, F., S. Chousterman, F. Horodniceanu, P. Kourilsky, and A.-C. Garapin.** 1979. Cloning of the active thymidine kinase gene of herpes simplex virus type 1 in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. **76**:3755–3759.
5. **Cronmiller, C., and B. Mintz.** 1978. Karyotypic normalcy and quasinormalcy of developmentally totipotent mouse teratocarcinoma cells. Dev. Biol. **67**:465–477.
6. **Cudennec, C. A., and G. R. Johnson.** 1981. Presence of multipotential hemopoietic cells in teratocarcinoma cultures. J. Embryol. Exp. Morphol. **61**:51–59.
7. **Cudennec, C. A., and J.-F. Nicolas.** 1977. Blood formation in a clonal cell line of mouse teratocarcinoma. J. Embryol. Exp. Morphol. **38**:203–210.
8. **Dewey, M. J., D. W. Martin, Jr., G. R. Martin, and B. Mintz.** 1977. Mosaic mice with teratocarcinoma-derived mutant cells deficient in hypoxanthine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U.S.A. **74**:5564–5568.
9. **Dierks, P., A. Van Ooyen, N. Mantei, and C. Weissmann.** 1981. DNA sequences preceding the rabbit β-globin gene are required for formation in mouse L cells of β-globin RNA with the correct 5′ terminus. Proc. Natl. Acad. Sci. U.S.A. **78**:1411–1415.
10. **Fritsch, E. F., R. M. Lawn, and T. Maniatis.** 1980. Molecular cloning and characterization of the human β-like globin gene cluster. Cell **19**:959–972.
11. **Kantor, J. A., P. H. Turner, and A. W. Nienhuis.** 1980. Beta thalassemia: mutations which affect processing of the β-globin mRNA precursor. Cell **21**:149–157.
12. **Lewis, W. H., P. R. Srinivasan, N. Stokoe, and L. Siminovitch.** 1980. Parameters governing the transfer of the genes for thymidine kinase and dihydrofolate reductase into mouse cells using metaphase chromosomes or DNA. Somat. Cell Genet. **6**:333–347.
13. **Linnenbach, A., K. Huebner, and C. M. Croce.** 1980. DNA-transformed murine teratocarcinoma cells: Regulation of expression of simian virus 40 tumor antigen in stem versus differentiated cells. Proc. Natl. Acad. Sci. U.S.A. **77**:4875–4879.
14. **Mantei, N., W. Boll, and C. Weissmann.** 1979. Rabbit β-globin mRNA production in mouse L cells transformed with cloned rabbit β-globin chromosomal DNA. Nature **281**:40–46.
15. **Mintz, B.** 1978. Gene expression in neoplasia and differentiation. Harvey Lect. **71**:193–246.
16. **Mintz, B.** 1979. Teratocarcinoma cells as vehicles for introducing mutant genes into mice. Differentiation **13**:25–27.
17. **Mintz, B., and C. Cronmiller.** 1981. METT-1: a karyotypically normal *in vitro* line of developmentally totipotent mouse teratocarcinoma cells. Somat. Cell Genet. **7**:489–505.
18. **Mintz, B., and K. Illmensee.** 1975. Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **72**:3585–3589.
19. **Mulligan, R. C., and P. Berg.** 1980. Expression of a bacterial gene in mammalian cells. Science **209**:1422–1427.
20. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U.S.A. **78**:2072–2076.
21. **Parker, B. A., and G. R. Stark.** 1979. Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. **31**:360–369.
22. **Pellicer, A., E. F. Wagner, A. El Kareh, M. J. Dewey, A. J. Reuser, S. Silverstein, R. Axel, and B. Mintz.** 1980. Introduction of a viral thymidine kinase gene and the human β-globin gene into developmentally multipotential mouse teratocarcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **77**:2098–2102.
23. **Perucho, M., D. Hanahan, and M. Wigler.** 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell **22**:309–317.
24. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
25. **Robins, D. M., S. Ripley, A. S. Henderson, and R. Axel.** 1981. Transforming DNA integrates into the host chromosome. Cell **23**:29–39.
26. **Segal, S., and G. Khoury.** 1979. Differentiation as a requirement for simian virus 40 gene expression in F-9 embryonal carcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **76**:5611–5615.

26a. **Stewart, T. A., and B. Mintz.** 1981. Successive generations of mice produced from an established culture line of euploid teratocarcinoma cells. Proc. Natl. Acad. Sci. U.S.A. **78**:6314–6318.

27. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
28. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
29. **Wagner, E. F., T. A. Stewart, and B. Mintz.** 1981. The human β-globin gene and a functional viral thymidine kinase gene in developing mice. Proc. Natl. Acad. Sci. U.S.A. **78**:5016–5020.
30. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
31. **Watanabe, T., M. J. Dewey, and B. Mintz.** 1978. Teratocarcinoma cells as vehicles for introducing specific mutant mitochondrial genes into mice. Proc. Natl. Acad. Sci. U.S.A. **75**:5113–5117.
32. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.
33. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11**:223–232.
34. **Wold, B., M. Wigler, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Introduction and expression of a rabbit β-globin gene in mouse fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **76**:5684–5688.